The Antitumor Agent CC-1065 Inhibits Helicase-Catalyzed Unwinding of Duplex DNA†

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ABSTRACT: The antitumor drug CC-1065 is thought to exert its effects by covalent bonding to N3 of adenine in DNA and interfering with some aspect of DNA metabolism. Therefore, it is of interest to determine what effect this drug has on enzymes involved in various aspects of DNA metabolism. In this report, we examine the ability of two DNA helicases, the dda protein of phage T4 and helicase II of *Escherichia coli*, to unwind CC-1065-adducted, tailed, oligonucleotides. It is shown that the presence of the drug on DNA strongly inhibits unwinding catalyzed by the T4 and *E. coli* proteins. A significant difference between the results obtained with the two helicases is that DNAs containing drug on either the tailed or the completely duplex strands are poor substrates for helicase II but dda protein-mediated unwinding is inhibited only when the drug is on the tailed strand. The drug-modified, helicase-released, strands migrate abnormally through a native gel, suggesting that the drug traps an unusual secondary structure generated in the course of protein-mediated unwinding. A kinetic analysis of the drug-inhibited reactions reveals that the helicases are trapped by the DNA–drug complex. This is evidenced by a decrease in the rate of helicase exchange between drug-bound substrate and drug-free duplex. The implications of these results with respect to the mechanism of action of CC-1065 in vivo are discussed.

The topological nature of the double helix presents a fundamental problem in DNA metabolism. While the helix must be relatively stable to help maintain the integrity of the genome while the cell is quiescent, DNA replication and a number of other processes require this structure to be unwound rapidly. This task falls to a special class of enzymes known as DNA helicases, which utilize energy derived from nucleotide triphosphate hydrolysis to fuel a unidirectional progression through the helix, thereby unwinding it. Although the mechanism of helicase-mediated unwinding is not known, the biochemical properties of numerous helicases from a variety of organisms have been elucidated. In vitro, many helicases, especially those involved in replication, efficiently unwind only substrates that include a single-stranded region to which the protein binds initially. The polarity of movement of any particular helicase is defined with respect to this initiating strand. In vivo, helicase loading may involve specific protein–protein interactions with other DNA binding factors.

Most in vitro studies of DNA helicases have understandably involved naked DNA substrates. However, there is ample reason for interest in modified substrates as well, since the in vivo substrate for these factors is probably very rarely naked DNA. One focus of study has been protein-bound templates (Bedinger et al., 1983; Bonne-Andrea et al., 1990). These reports have highlighted the importance of DNA helicases in dealing with these more biologically relevant substrates. For example, Alberts and co-workers have shown that a helicase encoded by the T4 dda gene is required in vitro for the phage DNA polymerase holoenzyme to proceed through a promoter-bound or moving *Escherichia coli* RNA polymerase and for the utilization of nucleosome-coated DNA as a template.

Chemically modified duplexes have been used extensively in studies of DNA polymerases and DNA repair proteins since such efforts are relevant to the ability of cells to deal with the toxicity and mutagenicity of various small molecules. However, there has been very little work with covalently modified DNAs that has focused on helicases. These studies are of interest because, according to current models of replication and recombination, the helicase is likely to be the first component of the "protein machines" (Alberts, 1984) that catalyze these processes to encounter modified substrates. If drugs such as CC-1065 (vide infra) completely inhibit unwinding of duplex DNA, any effect they might have, for example, on the DNA polymerase itself would be academic.

In this study, we employ DNAs modified with the DNA binding drug (+)-CC-1065. CC-1065 is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis* (Hanka et al., 1978; Warpehoski & Hurley, 1988; Reynolds et al., 1986). It is active against several experimental murine tumors in vivo and is about 100 times more potent than adriamycin against a broad spectrum of tumors in the cloning assay (Bhuyan et al., 1982). The CC-1065 molecule (Figure 1) consists of three repeating pyrroloindole moieties, one of which (the A subunit) contains a DNA-reactive cyclopropyl function. CC-1065 bonds covalently through N3 of adenine (Hurley et al., 1984) and lies within the minor groove of DNA, covering a 4 bp and a 1 bp region to the 5′- and 3′-side, respectively, of the covalently modified adenine (Scahill et al., 1990). Upon thermal treatment of CC-1065–(N3 adenine)–DNA adducts, cleavage of the N-glycosidic linkage and subsequent backbone breakage occur to the 3′-side of the covalently modified adenine to leave a 5′-phosphate on the 3′-side of the break and, presumably, a modified deoxyribose on the 5′-side (Reynolds et al., 1985) (Figure 1). This strand breakage assay has been used to determine the DNA sequence selectivity of CC-1065. Two subsets of consensus bonding sites

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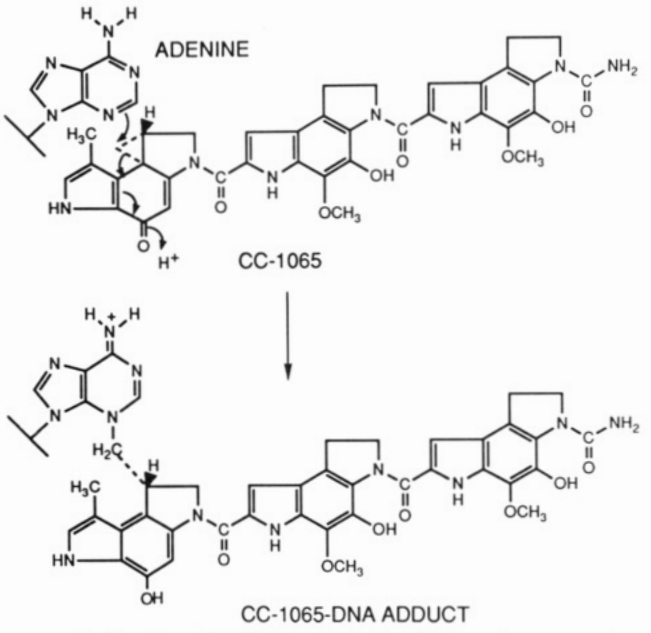

FIGURE 1: Reaction of CC-1065 with duplex DNA produces a covalent adduct.

(5′PuNTTA* and 5′AAAAA*, where the asterisk indicates the covalently modified adenine and N indicates any of the four bases in DNA) were identified initially (Reynolds et al., 1985), and later the consensus sequence 5′(A/T)(A/T)A* was found (Hurley et al., 1990). The construction of a site-directed CC-1065–(N3-adenine)–DNA adduct in a 117 bp fragment of M13mpl DNA (Needham-VanDevanter & Hurley, 1986) allowed determination of the effect of covalent attachment on local DNA structure. DNase I footprinting and restriction enzyme analysis demonstrated that CC-1065 adduct formation produces an apparent strand-selective and unidirectional effect on DNA structure, which extends more than one helix turn to the 5′-side of the covalent bonding site (Hurley et al., 1987). Upon covalent bonding to DNA, (+)-CC-1065 produces bending, winding, and stiffening of DNA (Lee et al., 1991).

The purpose of the work described here was to determine the effect of CC-1065 modification of DNA on the enzymatic processes involved in unwinding of DNA. Specifically, we have examined the ability of two DNA helicases, the bacteriophage T4 dda protein (Jongeneel et al., 1984) and the *E. coli* uvrD protein (helicase II) (Matson, 1986; Runyon & Lohman, 1989), to unwind tailed duplex oligonucleotides containing a CC-1065 binding site. We find that the presence of the drug inhibits unwinding catalyzed by the T4 and *E. coli* proteins. DNAs containing drug on either the tailed or the completely duplex strand are poor substrates for helicase II, but dda protein-mediated unwinding is inhibited only when CC-1065 is bound covalently to the tailed strand. The drug-modified, helicase-released, strands migrate abnormally through a native gel, suggesting that the drug traps an unusual secondary structure generated in the course of protein-mediated unwinding. A kinetic analysis of this process shows that the helicases are trapped by the drug–DNA complex, as evidenced by their inability to rapidly recycle to unwind an unmodified reporter duplex. Since the uvrD and dda proteins are known to be involved in DNA repair, replication, and recombination, these studies are relevant to the effect of CC-1065 and its analogues on many aspects of DNA metabolism.

Materials and Methods

Buffers. All buffers were made with double-distilled water. Annealing buffer is 100 mM NaCl/10 mM Tris-HCl (pH 8.0). DSC buffer is 15 mM NaCl/1.5 mM sodium citrate (pH 7.4). Helicase II unwinding buffer is 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 2.5 mM $MgCl_2$, 100 μg/mL bovine serum albumin (BSA), and 1.5 mM ATP. An ATP regeneration system including 10 mM creatine phosphate and 1 unit/mL creatine phosphokinase was also present. Dda unwinding buffer is 10 mM Tris–acetate (pH 7.4), 10 mM magnesium acetate, 1 mM dithiothreitol, 100 μg/mL BSA, and 90 mM KOAc. The ATP regeneration system was also included. Helicase stop buffer is 10 mM EDTA and 2% sodium dodecyl sulfate.

Table I: Tailed Duplex Constructs

Designation	Sequence
H1/L[a]	3′-ACTGGTACTAATGCCTAAGT-5′ 5′-TGACCATGATTACGGATTCATTTTTTTTTTTTTT-3′
H1/L/CC-1065[b]	3′-ACTGGTACTAATGCCTAAGT-5′ 5′-TGACCATGATTACGGATTCATTTTTTTTTTTTTT-3′
H1/S	3′-ACTTAGGCATTAGTACCAGT-5′ 5′-TGAATCCGTAATCATGGTCATTTTTTTTTTTTTT-3′
H1/S/CC-1065	3′-ACTTAGGCATTAGTACCAGT-5′ 5′-TGAATCCGTAATCATGGTCATTTTTTTTTTTTTT-3′
dda/L[c]	3′-ACTGGTACTAATGCCTAAGT-5′ 5′-TTTTTTTTTTTTTTTGACCATGATTACGGATTCA-3′
dda/L/CC-1065	3′-ACTGGTACTAATGCCTAAGT-5′ 5′-TTTTTTTTTTTTTTTGACCATGATTACGGATTCA-3′
dda/S	3′-ACTTAGGCATTAGTACCAGT-5′ 5′-TTTTTTTTTTTTTTTGAATCCGTAATCATGGTCA-3′
dda/S/CC-1065	3′-ACTTAGGCATTAGTACCAGT-5′ 5′-TTTTTTTTTTTTTTTGAATCCGTAATCATGGTCA-3′

[a] The designation H indicates that the single-stranded DNA is located to the 3′-side of the duplex region. [b] The designations L and S signify that the covalent binding site is one either the Long (35- or 51-nucleotide) or the Short (20-nucleotide) strand. The third position of the template designation indicates whether that construct contains bound CC-1065. [c] The dda designation denotes that the single-stranded tail is located on the 5′-side of the duplex region.

Proteins. Purified *Escherichia coli* uvrD gene product, helicase II, was a gift of Gregory T. Runyon and Prof. Timothy M. Lohman of Washington University. The dda protein was purified from *E. coli* S6934/dda/pTL. This strain, provided by Kevin Hacker and Prof. Bruce Alberts of the University of California at San Francisco, carries a plasmid containing the dda gene under the control of the λ P_L promoter and the cI857 gene. Expression and purification of the dda protein were carried out according to a procedure kindly provided by these workers prior to publication.

Chemicals. CC-1065 was isolated from the fermentation broth of *Streptomyces zelensis* (Hanka et al., 1978).

DNA Substrates. The oligonucleotides which were annealed to form the tailed duplexes shown in Table I were synthesized on an Applied Biosystems 381A instrument and purified by polyacrylamide gel electrophoresis. The strands designated long (L) and short (S) contain a 5 bp consensus binding site for CC-1065. DNA concentrations are given in terms of nucleotides.

Annealing was carried out by mixing approximately 5 μg of a 5′-^{32}P-labeled strand containing the CC-1065 binding site with a molar excess of the appropriate, unlabeled complementary strand in annealing buffer. The solution was heated to 55 °C and cooled slowly to 4 °C. The tailed duplex was separated from excess single-stranded oligonucleotide by electrophoresis through an 8% nondenaturing polyacrylamide

gel. The duplex-containing band was located by autoradiography, excised from the gel, and extracted with 800 μL of annealing buffer.

Drug binding was carried out by incubating gel-purified tailed duplex in annealing buffer with excess CC-1065 solution at room temperature for 3 days, as described previously (Lee et al., 1991). The drug-bound DNA was separated from excess drug by ethanol precipitation. The nomenclature we employ for the several substrates used in this study is given in Table I.

Thermal Cleavage Assay and DNA Sequencing. Drug-modified tailed duplexes were resuspended in 100 μL of DSC buffer and heated for 2 min at 95 °C to induce strand breakage on the 3′-side of the drug-adducted nucleotide (Reynold et al., 1985). The DNA samples were then denatured by heating for 3 min at 95 °C in an 80% formamide/10 mM NaOH solution and then electrophoresed through a 12% denaturing gel. The purine- and pyrimidine-specific Maxam–Gilbert sequencing reactions (Maxam & Gilbert, 1980) were loaded in adjacent lanes as markers.

Helicase II-Catalyzed Unwinding of CC-1065-Bound and Unbound Duplex DNA. Each 10-μL reaction was carried out in helicase II unwinding buffer containing 0.09 μM ^{32}P-labeled, drug-bound template. After preincubation for 5 min, an 8-fold molar excess of unlabeled 20mer or 35mer (whichever corresponds to the labeled strand) was added to the mixture as a trap to prevent the released, labeled strand from reannealing. The ratio of nucleotides (excluding the trapping DNA) to helicase II is given in the figure captions. Each reaction was incubated for 45 min at 30 °C and then quenched with helicase stopping buffer. After a further 15-min incubation to ensure protein denaturation, aliquots of the solutions were electrophoresed through a 15% native polyacrylamide gel at 5 V/cm for 6.5 h. The gel was dried onto DE-81 paper and the radioactive DNA visualized by autoradiography. The degree of unwinding was quantitated by densitometry of the autoradiogram.

Dda Protein-Catalyzed Unwinding of CC-1065-Bound and Unbound Duplex DNA. The experimental procedure was the same as for the helicase II unwinding reaction except for the buffer components and the DNA and protein concentrations. Each 20-μL reaction contained either 0.57 nM ^{32}P-labeled drug-bound substrate plus 2.8 nM 20 bp single-stranded oligonucleotide or 0.64 nM ^{32}P-labeled drug-bound template plus 6.3 nM 35 bp single-stranded oligonucleotide in dda unwinding buffer. In both the dda protein- and helicase II-mediated reactions, the excess unlabeled single-stranded oligonucleotide was added as a trap to prevent labeled strands from reannealing after helicase-mediated unwinding.

Phenanthroline–Copper Footprinting. Digestions of the uniquely ^{32}P-labeled (dda/S) duplexes, with and without drug, were performed according to the literature procedure (Yoon et al., 1988), except that they were carried out for 5 min at 20 °C. The substrates were then precipitated with ethanol, evaporated to dryness, dissolved in formamide/dye mixture, and loaded directly onto a 12% denaturing polyacrylamide gel.

The unlabeled duplex dda/S modified with CC-1065 was heat-denatured for 1 min at 95 °C, cooled quickly to 0 °C, and then loaded onto a 12% denaturing polyacrylamide gel to separate the strands. The band corresponding to the short strand containing the CC-1065–adenine adduct was visualized by UV fluorescence and excised from the gel, and the DNA was extracted with water. Finally, the extracted DNA was ethanol-precipitated, dried, resuspended in 20 μL of annealing buffer, and hybridized with the complementary ^{32}P-labeled long strand in order to generate duplex DNA. Sample prepared this way was then used in the phenanthroline–copper footprinting experiment together with the control duplex (dda/S) DNA modified with CC-1065.

Kinetic Study of Unwinding of Duplexes H1/L and H1/L/CC1065. Each reaction was carried out in 50 mL of helicase II unwinding buffer containing 5.5 μM DNA substrate (as nucleotides), and 0.12 or 0.06 μM helicase II for unwinding of H1/L/CC1065 or H1/L, respectively. After preincubation for 5 min, an 8-molar excess of unlabeled ssDNA trap (identical to ^{32}P-labeled strand) was added to the reaction. At given time intervals, 5 μL of sample was withdrawn and quenched with helicase stop buffer.

Effect of (+)-CC-1065-Modified Substrate on the Unwinding of Control Substrate Mediated by Helicase II. In order to investigate the effect of drug-modified substrate on the unwinding of "reporter" nonmodified substrate, reactions were carried out in 20 μL of helicase II unwinding buffer containing 2.8 μM H1/S as nucleotide and a given amount of helicase II with or without 1.4 μM H1/S/CC1065. Reactions were incubated for 45 min after a 5-min preincubation without 8-molar excess trap DNA.

Kinetic Study of the Unwinding of Dda/L in the Presence of Dda/L/CC1065. The 20-μL reactions were carried out in the dda unwinding buffer described previously. The first reaction consisted of 4.5 nM dda/L plus 33 nM trapping strand. The assay was started by the addition of 0.21 nM dda (4000:1 nucleotide:dda ratio). At the time points indicated in Figure 9, aliquots were removed from the reaction and mixed with helicase quenching buffer followed by freezing in liquid nitrogen. In the assay containing both templates, 0.63 nM dda was preincubated for 3 s with 5 nM dda/L/CC1065 plus 0.1 μM trapping strand. The reaction was started by adding 4.5 nM dda/L (4000:1 nucleotide:dda ratio) followed by the removal of aliquots as previously described. Finally, all of the aliquots representing different time points were electrophoresed through a 20% polyacrylamide gel and analyzed by autoradiography. Reactions in which quenching buffer was mixed with dda/L before the addition of dda showed no unwinding in 4 min (data not shown). Reactions consisting of only dda/L/CC1065 were not unwound at these concentrations of dda (data not shown).

Results

CC-1065 Adducts Inhibit Helicase II-Mediated Unwinding When Located on Either Strand. Our initial experiment tested whether CC-1065 covalently bound to DNA inhibits the unwinding of duplex DNA catalyzed by helicase II. Four duplex substrates containing a 3′-single-stranded poly(dT) tail were examined. The single-stranded DNA facilitates binding of the helicase to the substrate (Matson, 1986). Two of the tailed duplexes were drug-modified: one on the tailed strand (H1/L/CC1065) and one on the short strand (H1/S/CC1065) (Table I). The other two DNAs were identical to those described above, but did not contain the drug. In each case, only the strand with the CC-1065 binding site was labeled. Drug-bound or unmodified duplex DNA was mixed with the helicase at varying molar ratios of nucleotide to helicase (from 10:1 to 400:1). The amount of DNA, expressed as nucleotides, includes only the template and not the unlabeled trapping strand. Figure 2 shows that helicase II unwound the CC-1065-containing substates much less efficiently than the corresponding control DNAs. Under the conditions of these experiments, the control DNAs were unwound over the entire range of nucleotide:helicase II ratios investigated. However, both of the drug-containing substrates resisted unwinding by

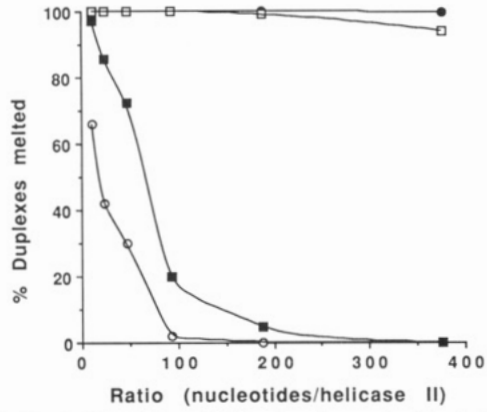

FIGURE 2: Unwinding of the tailed duplex oligonucleotides by helicase II is inhibited when CC-1065 is bound to either DNA strand. (■) H1/L/CC-1065; (○) H1/S/CC-1065; (□) H1/L; (●) H1/S. See Table I for substrate designations.

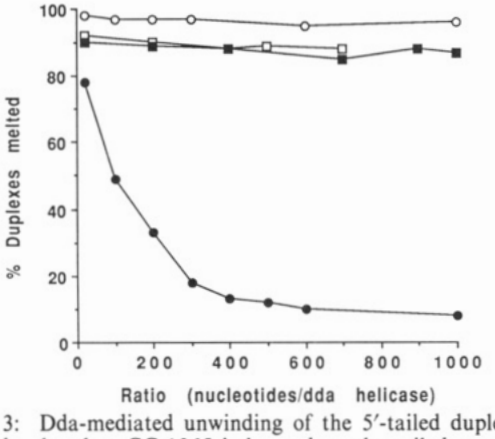

FIGURE 3: Dda-mediated unwinding of the 5′-tailed duplexes is inhibited only when CC-1065 is located on the tailed strand (●, dda/L/CC-1065). Binding of the drug to the opposite strand does not interfere with DNA unwinding (■, dda/S/CC-1065). This construct is melted as easily as the two control duplexes (○, dda/L, and □, dda/S).

helicase II. For example, less than 10% of the duplexes were melted at a nucleotide:helicase II ratio of 150:1. The degree of inhibition was somewhat greater when CC-1065 was bound to the short strand.

CC-1065 Inhibits Dda Protein-Catalyzed Unwinding Only When Bound to the Tailed Strand. To examine the effect of CC-1065 on the dda protein's unwinding activity, essentially the same experiments were carried out as were described above for helicase II. However, because the dda protein exhibits a 5′ to 3′ polarity (Jongeneel et al., 1984), opposite that of helicase II, the DNA substrates for the T4 helicase have 5′-tails (Table I). As in the helicase II-mediated reactions, the unmodified duplexes were melted efficiently at all protein:DNA ratios investigated, and the substrate containing the drug bound to the tailed strand was highly resistant to dda protein-catalyzed unwinding (Figure 3). However, the DNA in which the drug is bound to the short strand is an excellent substrate for the dda protein. Dda/S/CC1065 was melted by the dda protein as efficiently as the control substrates at all protein concentrations examined, in striking contrast to the corresponding helicase II result.

In order to make sure that CC-1065 was indeed bound to all of the drug-modified substrates, a thermal strand break experiment was performed using a procedure (see Materials and Methods) that cleaves the phosphodiester backbone of drug-bound strands on the 3′-side of the modified adenine. For each substrate, including dda/S/CC1065, complete conversion of the duplex DNA to strand break product was observed

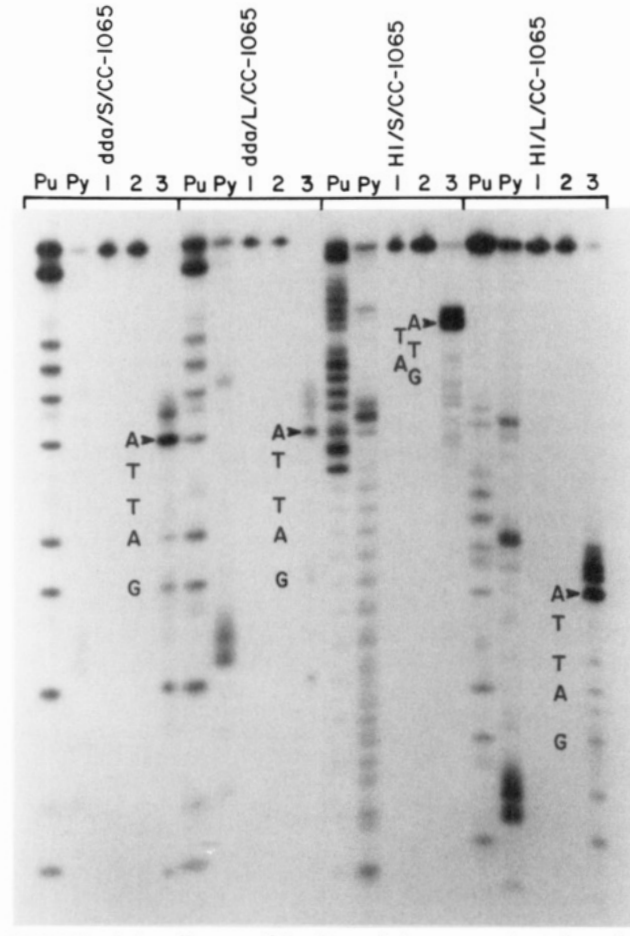

FIGURE 4: Autoradiogram of the thermal cleavage products from the partial duplex oligonucleotides modified with CC-1065. 5′-Labeled oligonucleotides, dda/S, H1/S, dda/L, and H1/L, were treated with 28 μM CC-1065, purified, subjected to strand break conditions as described under Materials and Methods, and electrophoresed through a 12% denaturing polyacrylamide gel. Pu and Py represent Maxam and Gilbert purine (Pu) and pyrimidine (Py) sequencing lanes. The arrowheads indicate the covalently modified adenines, followed by the remainder of the 5 bp drug recognition region. The samples in the numbered lanes of each of the groups are as follows: CC-1065 nonmodified DNA (lane 1); CC-1065-modified DNA without thermal treatment (lane 2); CC-1065-modified DNA with thermal treatment (lane 3).

(Figure 4). Therefore, the highly efficient unwinding of the dda/S/CC1065 substrate by the T4 helicase is not due to a low yield of drug bonding to this DNA.

To further substantiate the observation of strand-dependent inhibition of dda protein-catalyzed unwinding by CC-1065, we conducted unwinding experiments in which both drug-adducted, 5′-tailed substrates were present in the same tube. Two runs were done in which the nucleotide:helicase ratios were 20:1 and 230:1, respectively. If the data in Figure 3 are correct, we could expect dda/S/CC1065 to be unwound more efficiently than dda/L/CC1065 at the lower dda protein concentration, but both DNAs to be melted at the higher protein concentration. This is exactly the result observed (Figure 5, lanes 13 and 14).

Helicase-Released, Drug-Bound, Strands Run Anomalously on Native Gels. In the course of these investigations, it became obvious that the CC-1065-linked, helicase-released, strands migrate anomalously through native gels. For example, heat denaturation of the drug-adducted duplexes dda/S/CC1065 and dda/L/CC1065 produced two labeled single strands that have the expected electrophoretic migrations for a 20mer and 35mer (Figure 5, lane 9). These drug-bound DNAs comigrate

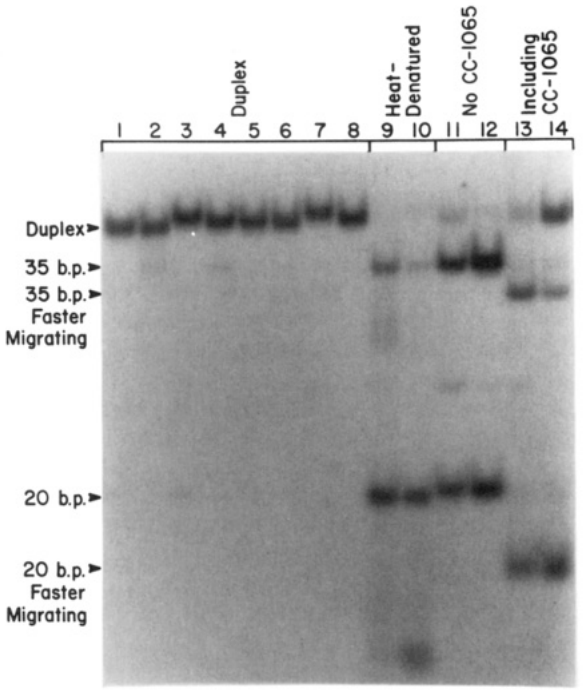

FIGURE 5: Dda protein-mediated unwinding of CC-1065–DNA complexes yields anomalously migrating single strands. The samples contained in each lane are as follows: dda/S (lane 1); dda/L (lane 2); H1/S (lane 3); H1/L (lane 4); dda/S/CC-1065 (lane 5); dda/L/CC-1065 (lane 6); H1/S/CC-1065 (lane 7); H1/L/CC-1065 (lane 8); heat-denatured dda/S/CC-1065 and heat-denatured dda/L/CC-1065 (lane 9); heat-denatured H1/S/CC-1065 and heat-denatured H1/L/CC-1065 (lane 10); dda/S and dda/L plus dda (0.72 μM DNA as nucleotides/1.3 nM dda) (lane 11); H1/S and H1/L plus helicase II (0.43 μM DNA as nucleotides/1.2 nM helicase II) (lane 12); dda/S/CC-1065 (labeled on the short strand) and dda/L/CC-1065 (labeled on the long strand) plus dda (0.2 μM DNA/11.0 nM dda) (lane 13); dda/S/CC-1065 and dda/L/CC-1065 plus dda (0.2 μM DNA/0.87 nM dda) (lane 14) (labels on the same strands as in the lane 13 experiment).

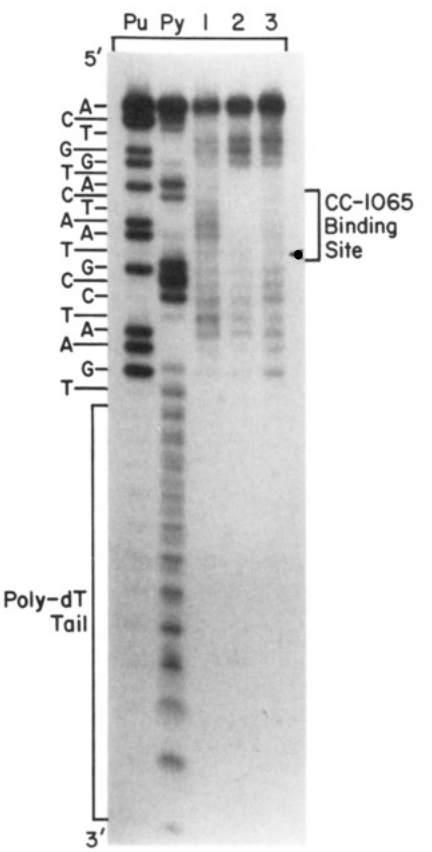

FIGURE 6: Phenanthroline–copper footprinting of reannealed dda/S/CC-1065 indicates that CC-1065 remains bound to the majority of template strands after a brief heat treatment. The arrowhead points to the complementary base of the drug-modified adenine. The bracket in lane 3 indicates the drug-overlap region. Pu and Py represent Maxam and Gilbert purine (Pu) and pyrimidine (Py) sequencing lanes. The samples in the numbered lanes are as follows: dda/S (lane 1); dda/S/CC-1065 (lane 2); reannealed dda/S/CC-1065 (lane 3).

with the strands released from heat denaturation of the unmodified substrates (not shown). Furthermore, bands of almost the same mobilities are produced when the unmodified substrates dda/L and dda/S are subjected to dda protein-catalyzed unwinding (Figure 5, lane 11). However, the bands representing the 20- and 35-nucleotide strands released by dda protein-mediated unwinding of the drug-adducted substrates dda/L/CC1065 and dda/S/CC1065 run much faster (Figure 5, lane 13). Interestingly, when these fast-running DNAs are isolated from the gel, heated briefly at 95 °C, cooled, and electrophoresed again, they no longer migrate anomalously (not shown).

This heat-induced return to a normal electrophoretic migration is not due to loss of the drug during the heating step. This was demonstrated by examining the hydroxyl radical cleavage pattern of a tailed duplex constructed by annealing a drug-bound strand that had been subjected to heating with the appropriate complementary strand. The presence of covalently bound CC-1065 significantly inhibits cleavage in the region of drug binding (D. K. Sun and L. H. Hurley, unpublished results). Thus, the degree of protection of the reconstituted substrate is a sensitive indicator of the continued presence of the drug. Specifically, unlabeled substrate dda/S/CC1065 was heat-denatured by 95 °C, and the strands were separated by preparative polyacrylamide gel electrophoresis. The purified 20-nucleotide strand was then reannealed to its ^{32}P-labeled complementary 35-nucleotide strand. The reconstituted dda/S/CC1065 substrate was then subjected to copper–phenanthroline hydroxyl radical footprinting. The cleavage pattern exhibited by the reannealed template is shown in Figure 6, lane 3. Also shown in Figure 6 are the cleavage patterns observed for the unmodified substrate dda/S (lane 1) and a sample of dda/S/CC1065 that had not been subjected to heat treatment. Comparison of lanes 1 and 2 demonstrates the high degree of protection afforded by the bound drug. A considerable degree of protection is also seen in lane 3, leading us to conclude that the majority of the reconstituted dda/S/CC1065 molecules retain covalently-bound drug. The weak cleavage pattern observed in the region of drug binding suggests that a small fraction of molecules lose the drug upon heat treatment or isolation from the polyacrylamide gel. A second experiment was also performed to test for the presence of the drug on the helicase-released bands. Helicase II-released, drug-bound, DNA was isolated from a native polyacrylamide gel after having been heated for 1 min in order to induce a normal rate of migration. This sample was then subjected to the thermal strand break reaction; 97% of the DNA was cleaved at the expected position (data not shown). Taken together, the experiments discussed above lead us to conclude that the rapid migration of the drug-adducted, helicase-released strands is due to some type of drug-stabilized secondary structure induced in the course of helicase-mediated unwinding. This interpretation is strengthened by the observation that fast-migrating bands are also produced by helicase II-catalyzed unwinding of the 3′-tailed, drug-adducted, substrates H1/S/CC1065 and H1/L/CC1065 (not shown).

Helicases Are Trapped Kinetically by the Drug–DNA Complex. In order to probe the mechanism of inhibition, we

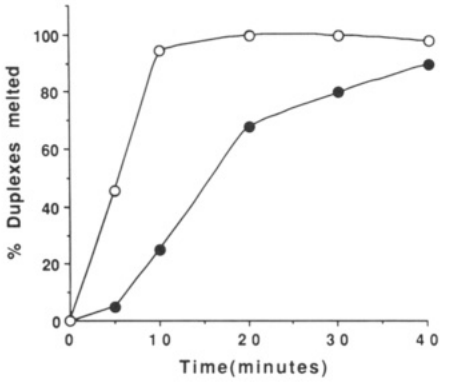

FIGURE 7: Helicase II unwinds the drug-adducted substrates (H1/L/CC1065, ●) more slowly than the unmodified substrate (H1/L, ○).

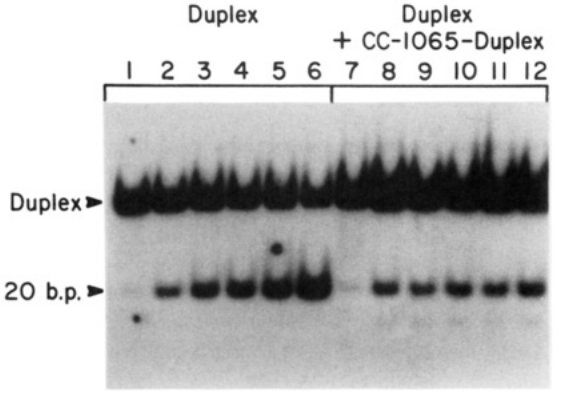

FIGURE 8: CC-1065 adduct traps the uvrD protein as evidenced by its inability to recycle and melt an unmodified "reporter" duplex. All reactions were carried out as described under Materials and Methods. Lanes 1–6, 2.8 μM of H1/S as nucleotides; lane 7–12, 2.8 μM H1/S and 1.4 nM H1/S/CC1065. Lanes 1 and 7, no helicase II; lanes 2 and 8, lanes 3 and 9, lanes 4 and 10, lanes 5 and 11, and lanes 6 and 12 contained 1.4, 7, 14, 28, and 70 nM helicase II, respectively.

examined the kinetics of the unwinding reactions. We chose a helicase II to DNA ratio that would eventually allow considerable unwinding of both the nonmodified and modified duplexes. Figure 7 shows that the unmodified substrate is unwound more rapidly than the CC-1065-bound duplex. For example, after a 10-min incubation, the former was melted completely while less than 25% of the drug-modified duplex was unwound.

One possible interpretation of this result is that helicase II moves very slowly through the region to which the drug is bound. An alternative explanation is that when the enzyme encounters the drug, it falls off the DNA and must reinitiate. In this model, only a minority of binding/unwinding cycles proceed through the CC-1065-modified region. To distinguish between these models, we evaluated the ability of helicase II to unwind unmodified DNA in the presence of a drug-modified substrate. If the recycling model is correct, then the unmodified "reporter" substrate should be unwound rapidly by the helicase that has fallen off of the drug-modified DNA. As shown in Figure 8, this is not the case. In one experiment, we incubated varying amounts of helicase II (see figure caption) with 2.8 μM ^{32}P-labeled H1/S as nucleotide (Table I). As expected, the amount of DNA unwound after a 45-min incubation increased with increasing helicase concentration. In a parallel experiment, identical incubations were performed except that 1.4 μM CC-1065-adducted H1/S (as nucleotides) was also present. In this case, the amount of DNA unwound was uniformly low at each concentration of helicase. Since both the drug-bound and unmodified DNAs were added si-

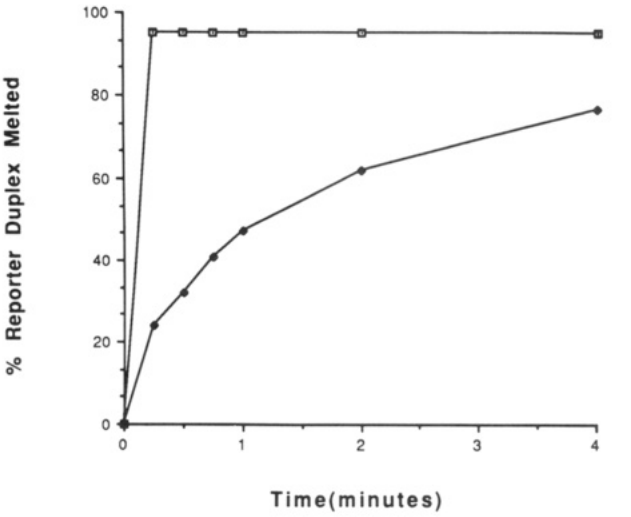

FIGURE 9: Dda helicase is stalled by the CC-1065 DNA adduct as evidenced by slow unwinding of a reporter duplex in the presence of the modified oligonucleotide. Complete unwinding of non-drug-bound duplex DNA occurs in less than 15 s (⊡). Melting this same duplex in the presence of enough dda/L/CC1065 to bind all of the dda requires more than 4 min (♦). The nucleotide:dda ratios in the two reactions are equivalent.

multaneously, we believe that the unwound DNA in the latter experiment was due to helicase molecules that encountered the unmodified substrate first. We conclude from the data shown in Figures 7 and 8 that helicase II is trapped by the DNA–drug adduct. The stalled enzyme is unable to recycle rapidly.

To demonstrate that CC-1065 interferes with the movement of dda, an experiment similar to that shown in Figure 8 was performed. The helicase was added to a mixture of both dda/L/CC1065 and dda/L at a concentration that is sufficient to unwind naked, but not melt CC-1065-bound, duplexes. The "reporter" substrate in this assay was the ^{32}P-labeled unmodified duplex. Dda/L alone was unwound completely 15 s after the addition of dda (the earliest time point). Dda/L/CC-1065 slowed down the unwinding of dda/L when it was added to the reaction mixture (Figure 9), although the reporter duplex is eventually unwound. Thus, both helicases are trapped kinetically by the CC-1065–DNA block, but the dda protein releases more rapidly than does the uvrD protein. This difference is in accord with previous observations which demonstrated that the uvrD protein is highly processive but the dda protein is not.

Discussion

The antitumor drug CC-1065 is thought to exert its biological effects by bonding covalently to double-stranded DNA and presumably interferes with one or more aspects of cellular DNA metabolism. There is currently little known about its effect on the activities of various proteins involved in these processes. In order to begin to address such questions, we have investigated the effect of CC-1065 and some of its analogues on the unwinding reactions catalyzed by two DNA helicases: the dda protein of phage T4 and the uvrD protein (helicase II) of *E. coli*. We are particularly interested in the ability of DNA helicases to deal with drug-adducted substrates for a number of reasons. Replicative helicases are thought to be located in the vanguard of the multiprotein complexes that catalyze DNA synthesis and play a crucial role in providing the DNA polymerase with a single-stranded template that can be utilized efficiently (Alberts, 1984). Therefore, the helicase would be the first component of the replication complex to

encounter the CC-1065-modified DNA. If the drug, which is known to stabilize a duplex, interferes with helicase-mediated unwinding, one would predict that this would have severe consequences for further fork progression. In addition, other major pathways of DNA metabolism are known to utilize DNA helicases, such as homologous recombination and DNA repair. Therefore, the ability of helicases to melt CC-1065-adducted substrates is relevant to the effect the drug may have on these processes as well.

In order to keep the system as simple as possible, we opted to examine as substrates short, tailed, drug-modified duplexes constructed from synthetic oligonucleotides. The choice of the dda and uvrD proteins as the particular helicases to be studied was due to the fact that dda is thought to function in phage recombination and replication (Bedinger et al., 1983; Formosa & Alberts, 1986; Kodadek & Alberts, 1987; Kodadek, 1991), while helicase II is a DNA repair enzyme. Therefore, information relevant to diverse aspects of DNA metabolism can be obtained.

While CC-1065 exerts its effects in eukaryotic cells, we have utilized two helicases isolated from prokaryotic organisms. This is partly because these enzymes are much more readily available and better characterized than any of their eukaryotic counterparts. In addition, the major mechanisms of DNA metabolism have been generally conserved through evolution, and we assume our results are representative of the events that occur in a eukaryotic cell exposed to CC-1065.

The data in Figures 2 and 3 show clearly that CC-1065 does indeed inhibit helicase-mediated unwinding of duplex DNA relative to unmodified substrates. In the case of helicase II-catalyzed reactions (Figure 2), the drug does not absolutely block unwinding at high protein concentrations, but the levels of enzyme required to observe helix melting are much higher than for identical reactions containing control substrates. Inhibition is observed when the drug is bound both to the tailed and to the completely duplex strand, although the effect is somewhat more pronounced in the former case.

The dda protein-catalyzed unwinding reactions are inhibited only when CC-1065 is attached to the tailed strand (Figure 3). The construct in which the drug is bound to the completely duplex strand (dda/S/CC-1065) is unwound by dda as efficiently as a control substrate lacking the drug. The drug appears to stall the helicases without causing them to fall off of the duplex. This is evidenced in the helicase II-mediated reactions by the huge lag period observed in the time course of protein-catalyzed unwinding of the drug-bound DNAs (Figure 7) and the inability of the uvrD protein to unwind an unmodified reporter duplex in the presence of the DNA–drug adduct (Figure 8).

Both helicases operate less efficiently on substrates in which the drug is located on the tailed strand. Why is the degree of inhibition so much more strand-dependent in the case of dda protein-mediated reactions? Since there is so little known about the molecular details of helicase-catalyzed duplex unwinding, it is difficult to address this point in a rigorous manner. One possibility is that the uvrD protein associates with both strands during the unwinding reaction while the dda protein is much more strongly associated with the strand to which it binds initially. Another is that the helicases for some reason have more difficulty proceeding through the drug–DNA complex when they encounter it from the 5′-side. This could be due to the recently discovered winding effect CC-1065 has on the duplex, which is propogated much more strongly to the 5′-side of the modified adenine than the 3′-side (Lee et al., 1990; Sun and Hurley 1992, unpublished results). If both of the aforementioned models contribute to inhibition of unwinding, this would help rationalize the much greater strand dependence observed in the dda protein experiments. Dda/L/CC-1065, which is highly resistant to melting, presents the 5′-side of the drug to the approaching helicase *and* has the drug bound to the strand on which the helicase is running. However, because of the opposite polarity of helicase II movement, these effects are not compounded in the helicase II substrates. H1/L/CC-1065 has the drug bound to the tailed strand, but the 5′-end of the drug points away from the approaching helicase. H1/S/CC-1065 presents the 5′-side of the drug to the helicase, but does not have the drug linked covalently to the tailed strand.

A curious feature of the single-stranded, CC-1065-modified, fragments released by the helicases is that they migrate anomalously through native gels. Both helicases produce products with identical mobilities. Brief heating of these species induces a return to a normally migrating band. Several lines of experimental evidence demonstrate that the drug remains bound to the DNA after helicase-mediated unwinding and heat treatment. The simplest explanation of these data is that the helicases induce a peculiar secondary structure in the DNA during the unwinding reaction that is trapped by CC-1065. This kinetic product collapses to the thermodynamic product (the normal migrating species) upon heat treatment. This idea is in accord with the strong preference of CC-1065 for duplex binding, a property that would lead one to predict that CC-1065 would stabilize secondary structures. At present, we have no information regarding the nature of the helicase-induced structure.

What are the biological implications of these observations? Since the degree of inhibition of unwinding is dependent on the helicase:DNA ratio and these ratios are not known with precision in vivo, this is a difficult point to address. Nonetheless, it seems likely that CC-1065-adducted DNA is a poor substrate for any process that requires duplex unwinding, which is to say most aspects of DNA metabolism. Not only are modified duplexes harder to unwind, but also the single-stranded products released at high helicase concentrations have an unusual drug-stabilized secondary structure that in itself could have mutagenic or cytotoxic effects. Thus, the extreme toxicity of the drug may be due to interference with any one of a number of pathways. Studies with other enzymes involved in DNA replication, recombination, and transcription are underway to test these ideas further.

ACKNOWLEDGMENTS

We thank a referee for suggesting the kinetic studies.

REFERENCES

Alberts, B. M. (1984) *Cold Spring Harbor Symp. Quant. Biol. 49*, 1–12.

Bedinger, P., Hochstrasser, M., Jongeneel, C. V., & Alberts, B. M. (1983) *Cell 34*, 115–123.

Bonne-Andrea, C.; Wong, M. L., & Alberts, B. M. (1990) *Nature 343*, 719–726.

Brahme, N. M. (1990) *Biochemistry 29*, 2852–2860.

Formosa, T., & Alberts, B. M. (1986) *Cell 47*, 793–806.

Hanka, L. J., Dietz, A., Gerpheide, S. A., Kuenzel, S. L., & Martin, D. G. (1978) *J. Antibiot. 31*, 1211–1217.

Hurley, L. H., Reynolds, L. L., Swenson, D. H., & Scahill, T. (1984) *Science 226*, 843–844.

Hurley, L. H., Warpehoski, M. A., Lee, C.-S., McGovern, J. P., Scahill, T. A., Kelly, R. C., Mitchell, M. A., Wicnienski, N. A., Gebhard, I., Johnson, P. D., & Bradford, V. S. (1990) *J. Am. Chem. Soc. 112*, 4633–4649.

Jongeneel, C. V., Formosa, T., & Alberts, B. M. (1984) *J. Biol. Chem. 259*, 12925-12932.
Kodadek, T. (1991) *J. Biol. Chem. 266*, 9712-9718.
Kodadek, T., & Alberts, B. M. (1987) *Nature 326*, 312-314.
Lee, C. S., Kizu, R., Sun, D., & Hurley, L. H. (1991) *Chem. Res. Toxicol. 4*, 203-213.
Matson, S. W. (1986) *J. Biol. Chem. 261*, 10169-10175.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 498-560.
Reynold, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6628-6237.
Runyon, G. T., & Lohman (1989) *J. Biol. Chem. 264*, 17502-17512.
Scahill, T. A., Jensen, R. M., Swenson, D. H., Hatzenbuhler, N. T., Petzold, G. L., Sun, D., & Hurley, L. H. (1992) *Anticancer Drug Design* (in press).
Yoon, C., Kuwabara, M. D., Law, R., Wall, R., & Sigman, D. S. (1988) *J. Biol. Chem. 263*, 8458-8463.

Replication Inhibition and Translesion Synthesis on Templates Containing Site-Specifically Placed *cis*-Diamminedichloroplatinum(II) DNA Adducts†

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ABSTRACT: A series of site-specifically platinated, covalently closed circular M13 genomes (7250 bp) was constructed in order to evaluate the consequences of DNA template damage induced by the anticancer drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Here are reported the synthesis and characterization of genomes containing the intrastrand cross-linked adducts *cis*-[Pt(NH_3)$_2${d(ApG)-*N*7(1),-*N*7(2)}], *cis*-[Pt(NH_3)$_2${d(GpCpG)-*N*7(1),-*N*7(3)}], and *trans*-[Pt(NH_3)$_2${d(CpGpCpG)-*N*3(1),-*N*7(4)}]. These constructs, as well as the previously reported M13 genome containing a site-specifically placed *cis*-[Pt(NH_3)$_2${d(GpG)-*N*7(1),-*N*7(2)}] adduct, were used to study replication in vitro. DNA synthesis was initiated from a position approximately 177 nucleotides 3′ to the individual adducts, and was terminated either by the adducts or by the end of the template, located approximately 25 nucleotides on the 5′ side of the adducts. Analysis of the products of these reactions by gel electrophoresis revealed that, on average, bypass of the *cis*-DDP adducts occurred approximately 10% of the time and that the *cis*-[Pt(NH_3)$_2${d(GpG)-*N*7(1),-*N*7(2)}] intrastrand cross-link is the most inhibitory lesion. The *cis*-[Pt(NH_3)$_2${d(GpCpG)-*N*7(1),-*N*7(3)}] adduct allowed a higher frequency of such translesion synthesis (ca. 25%) for two of the polymerases studied, modified bacteriophage T7 polymerase and *Escherichia coli* DNA polymerase I (Klenow fragment). These enzymes have either low (Klenow) or no (T7) associated 3′ to 5′ exonuclease activity. Bacteriophage T4 DNA polymerase, which has a very active 3′ to 5′ exonuclease, was the most strongly inhibited by all three types of *cis*-DDP adducts, permitting only 2% translesion synthesis. This enzyme is therefore recommended for replication mapping studies to detect the location of *cis*-DDP–DNA adducts in a heterologous population. The major replicative enzyme of *E. coli*, the DNA polymerase III holoenzyme, allowed <10% adduct bypass. Postreplication restriction enzyme cleavage studies established that the templates upon which translesion synthesis was observed contained platinum adducts, ruling out the possibility that the observed products were due to a small amount of contamination with unplatinated DNA. The effects on in vitro replication of a recently characterized adduct of *trans*-DDP [Comess, K. M., Costello, C. E., & Lippard, S. J. (1990) *Biochemistry 29*, 2102-2110] were also evaluated. This adduct provided a poor block both to DNA polymerases and to restriction enzymes. The properties of this adduct in the M13 genome were investigated by postreplication sequence analysis of the translesion synthesis product. Taken together, these studies demonstrate that polymerases can traverse through all of the major bifunctional cisplatin adducts formed in vitro and in vivo and strengthen the hypothesis that adduct-induced mutagenesis may occur through replication bypass.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DPP),[1] as well as the clinically inactive isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP), form bifunctional covalent adducts with the nucleobases of DNA. These interactions are believed to contribute to cytotoxicity by inhibiting replication or transcription [general reviews: Fichtinger-Schepman et al. (1986), Umapathy (1989), and Bruhn et al. (1990)]. Studies of the biological responses to platinum(II) adducts have been restricted primarily to the full spectrum of adducts produced by platination of a heterogeneous population of DNA sequences, previously termed "global

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[1] Abbreviations: *cis*-DPP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; RE, restriction endonuclease; RF, replicative or double-stranded form of M13 genomes; ss, single-stranded or viral form of M13 genomes (this strand is always the plus strand); EDTA, ethylenediaminetetraacetate dianion; PNK, polynucleotide kinase; ATP, adenosine triphosphate; TE, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA; HAP, hydroxylapatite; T7 pol, modified bacteriophage T7 DNA polymerase (Sequenase 2.0); Taq pol, *Thermus aquaticus* DNA polymerase; T4 pol, bacteriophage T4 DNA polymerase; pol I, *Escherichia coli* DNA polymerase I large fragment; pol III, *Escherichia coli* DNA polymerase III holoenzyme.

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